ANALYSIS OF RAT BLOOD SAMPLES FOR AGENT BIOMARKERS AFTER GB INHALATION EXPOSURE

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ABSTRACT

A method was developed for the analysis of a GB nerve agent biomarker in blood that is very sensitive, selective, and applicable for archived samples. The biomarker resulting from sample acidification and in the presence of fluoride ion was regenerated GB (rGB) which was found in rat blood after inhalation exposures ranging from miosis to lethal levels. The method utilized a C18 solid-phase extraction (SPE) followed by quantification using a gas chromatograph with either a flame photometric detector (GC-FPD) or a mass spectrometer (GC-MS). Samples were concentrated by injecting the SPE extract on a Tenax -TA sorbent tube along with 100 pg of decadeuterated diethyl ethyl phosphonate as the internal standard followed by thermal desorption GC-FPD analysis and GC-MS confirmation. The method detection limit was 6 pg of agent and the working range was 20-200 pg GB on column. Quality control samples were analyzed and yielded spike recoveries greater than 95%.

INTRODUCTION

There is the need to be able to verify and model nerve agent exposure for medical, tactical, and political reasons. Current methods such as those based on cholinesterase activity are inadequate. Symptoms of exposure are not specific to the chemical warfare agents. Our objective was to find and/or develop analytical methods capable of quantifying low level nerve agent inhalation exposure in biological matrices such as blood and tissue using available instrumentation. The strategy to obtain the needed methods could be summarized in three steps which were: 1) Search the literature for potential methods, 2) Further develop methods as necessary, and 3) Validate using agent spiked matrices and inhalation exposure samples.

The literature search for nerve agent methods produced one very promising candidate method which served as a starting point. In this method, GB was regenerated from bound sites in the serum/plasma by relatively simple sample matrix manipulation followed by solid-phase cartridge extraction (Polhuijs et al. Toxicology and Applied Pharmacology 146, 156-161, 1997). This method served as a starting point. Modifications were made to the method in accordance with the instrumentation, standards, and objectives of our laboratory and research goals.

EXPERIMENTAL

MATERIALS

GB and VX were CASARM grade prepared and analyzed at ECBC and diluted with isopropyl alcohol or hexane. Decadeuderated diethyl ethylphosphonate ($^{2}H_{10}$DEEP)were synthesized at ECBC using an mixture of ethylphosphonic dichloride, Ethyl-$^{2}H_{5}$-alcohol, and N,N-diisopropylethylamine (Aldrich, Milwaukee, WI) in a 1:2:2 molar ratio, respectively, in acetonitrile, cold filtered to remove the resulting amine hydrochloride, and analyzed by GC-FPD and GC-MSD. The C_{18} SPE cartridges were 200mg and 500 mg (Waters Associates, Millipore Corp., Milford, MA) capacity. Acetate buffer

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(pH3.5)was prepared from 5.41 mL glacial acetic acid and 0.4435 g sodium acetate diluted to 500 mL with deionized water. Potassium fluoride was ACS reagent grade (Aldrich, Milwaukee, WI) dilute with deionized water to approximately 2 M. All other chemicals were procured commercially at ACS reagent grade or higher.

SAMPLE PREPARATION

Serum (human, guinea pig, rat sera from Sigma, St.Louis, MO) was spiked with dilute GB or VX to 1 ug/mL. The exposed sera were filtered using 500 mg C_{18} SPE cartridges to separate the free from the bound nerve agent. The free agent was eluted with 1 mL ethyl acetate, collected over sodium sulfate and saved for analysis. The resulting positive control sera was then analyzed for regenerated agent using acetate buffer and fluoride ion. For inhalation exposure samples, whole blood from GB exposed rats was collected (with and without EDTA) and centrifuged at 15,000 rpm for 3 min. The resulting cell pack and serum/plasma was analyzed for regenerated agent by the addition of acetate buffer and fluoride ion. Archived sample from 1998 were in the form of packed cells. The sample preparation steps were as follows :

- 1) Weigh Sample (0.1-0.5 g)
- 2) Add and mix(vortex): 1.5 mL acetate buffer pH 3.5, and 0.02 mL(plasma) or 0.4 mL (packed cells) of KF solution.
- 3) For packed cell samples centrifuge 4000 rpm for 10 min,
- 4) Transfer liquid to conditioned C18 SPE column (conditioned with 1 mL isopropanol followed by 1 mL acetate buffer),
- 5) Elute with 1-1.5 mL ethyl acetate over sodium sulfate
- 5m) For miosis levels: concentrate ethyl acetate to ~100uL
- 6) Spike 0.010-0.200 mL ethyl acetate on DAAMS tube (Tenax-TA), and then spike tube with internal standard (100 pg of $^{2}H_{10}$-DEEP), flush with N_2 for 3 min at 75 cc/min
- 6m) For miosis levels:spike entire contents of vial and wash vial with 2x50uL of ethyl acetate adding wash to DAAMS tube

INSTRUMENTAL

Samples were analyzed on either a Hewlett-Packard 5890 GC-FPD (dual flame photometric detectors) or a Hewlett-Packard 6890 GC-5973 MSD (Newark, DE). Sample inlet was by Tenax® solid sorbent tube (Depot Area Agent Monitoring System (DAAMS) tube: Dynatherm Inc, Oxford, PA) using an ACEM 900 (Dynatherm Inc, Oxford, PA) desorber interfaced to the GC column via butt-connector. The GC column was a 30 m x 25 mm x 0.5 um thickness DB-5 MS (J&W Scientific, Avondale, CA). The ACEM 900 temperature program was as follows: Dry 60 ^{o}C for 1 minute, Tube Heat 200^{o}C for 3minutes, Cool for 1minute, Trap Heat 275 ^{o}C for 3 minutes. The GC oven temperature program was as follows: Initial 40 ^{o}C for 2 minutes, ramp to 160 ^{o}C at 15 ^{o}C/minute, ramp to 260 ^{o}C at 40^{o}C/minute and held for 3 minutes. The MSD was used in the electron ionization mode with selected ion monitoring at m/z 81, 99, 125. After the sample was desorbed on the GC column the sorbent tube was reconditioned by backflushing using 100 mL/min flow of dry nitrogen at 280-300 ^{o}C for five to eight minutes to decrease the high boiling point interference from the serum samples. Backflushing of the sorbent tube prevents degradation of the instrument and column producing a stable baseline despite the complex nature of the sample matrix.

RESULTS

The GC-FPD or MSD(EI) with Dynatherm inlet to allows injection volumes from 1-400 uL(above 200 uL in MS). Volumes above 200 uL tended to decrease FPD sensitivity but were useful for GC-MSD analysis in some cases. The working range was 20-200 pg GB on column. The method detection limit was 0.006 ng/mL for GB(FPD). Positive controls were developed for human, guinea pig, and rat blood cells and serum. The extraction efficiency was determined to be better than 90%. The optimum levels of fluoride needed for regeneration varied among species and types of sample (serum vs blood cell fraction). VX-G which could represent an interference was baseline resolved from GB as shown in Figure 1.

GB inhalation rat packed cell samples stored at $5^{o}C$ for a year produced regenerated GB in the range of 1-10 ng/g with a mean of 4.88 ng/g and a standard deviation of 2.28 (n=38). Pre-exposure samples produced no GB. Immediately analyzed GB inhalation rat packed cell samples produced regenerated GB in the range of 2-36 ng/g with a mean of 18.6 ng/g and a standard deviation of 8.08 (n=48). Again, pre-exposure samples produced no GB. Serum samples that were analyzed soon after exposure produced regenerated GB in the range of 0.4-8 ng/g with a mean of 2.0 and standard deviation of 1.7 (n=11). Packed cell concentrations of regenerated GB were typically greater than serum/plasma levels at the higher Ct exposures, as shown in Figure 2

The mean percent GB standard spike recovery [(Found GB/Target level)100] was 98.3 +/- 11.2% (n=10). The mean percent GB standard matrix spike recovery (on-tube spikes) mean was 97.3 +/-7.44% (n=6). In general, regenerated GB levels in serum were lower than the cell fraction. Also, preliminary data from LCt50 level exposures indicated that female rats produce higher initial blood levels of regenerated GB than males depending on exposure level. However, after 14 days male and female levels appear similar, Figure 3.

DISCUSSION

The results indicate that GB can be verified from blood and tissue samples using GC-FPD or GC-MSD. In LCt50 experiments, recovered quantities of GB in samples from the cell fraction consistently exceeded by a factor of four or more those seen in the serum/plasma. This is possibly due to the presence of other binding sites such as carboxyesterases or possibly non-esterase related sites. Miosis level samples showed relative amounts of GB were greater in plasma/serum then packed red cells by a factor of six. Overall, there appears to be a better relationship between Ct and rGB concentration than Ct and cholinesterase activity given the data analyzed, Figures 4 and 5.

CONCLUSIONS

Methods for the quantification of low-level chemical nerve agent biomarkers after inhalation exposure has been developed that will allow exposure verification and blood/tissue concentration to be determined. The advantages are simplicity and speed of analysis, the ability to look at archived samples for evidence of exposure, applicability to all G and V agents, detection limits lower than miosis level exposure, and yield more information than cholinesterase-based metrics. The major disadvantage is that the original leaving group of the agent is not known but this is also true in the case of using agent metabolites as indicators of exposure.

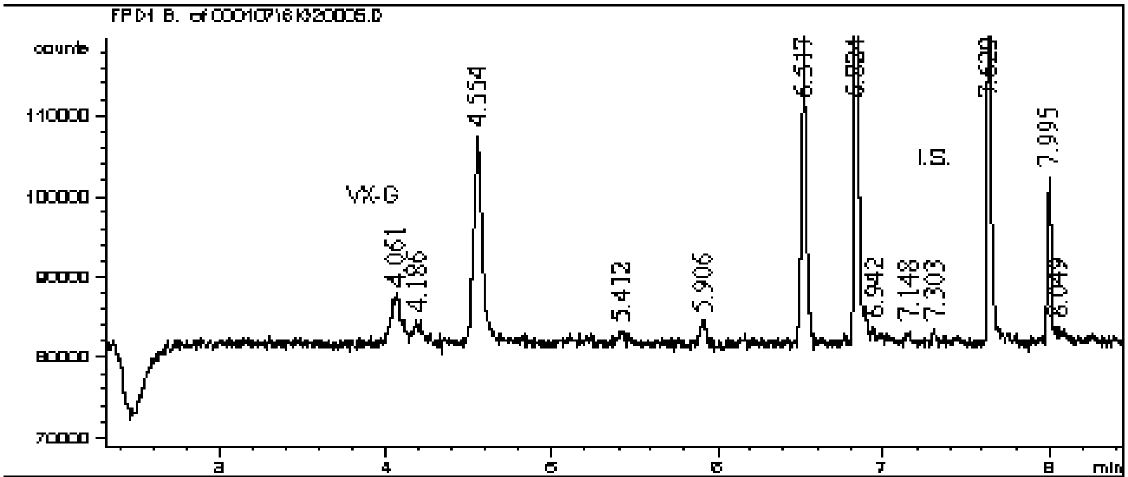

Figure 1. Chromatogram of VX-G(4.039 min) Spiked GB(4.535 min) Sample.

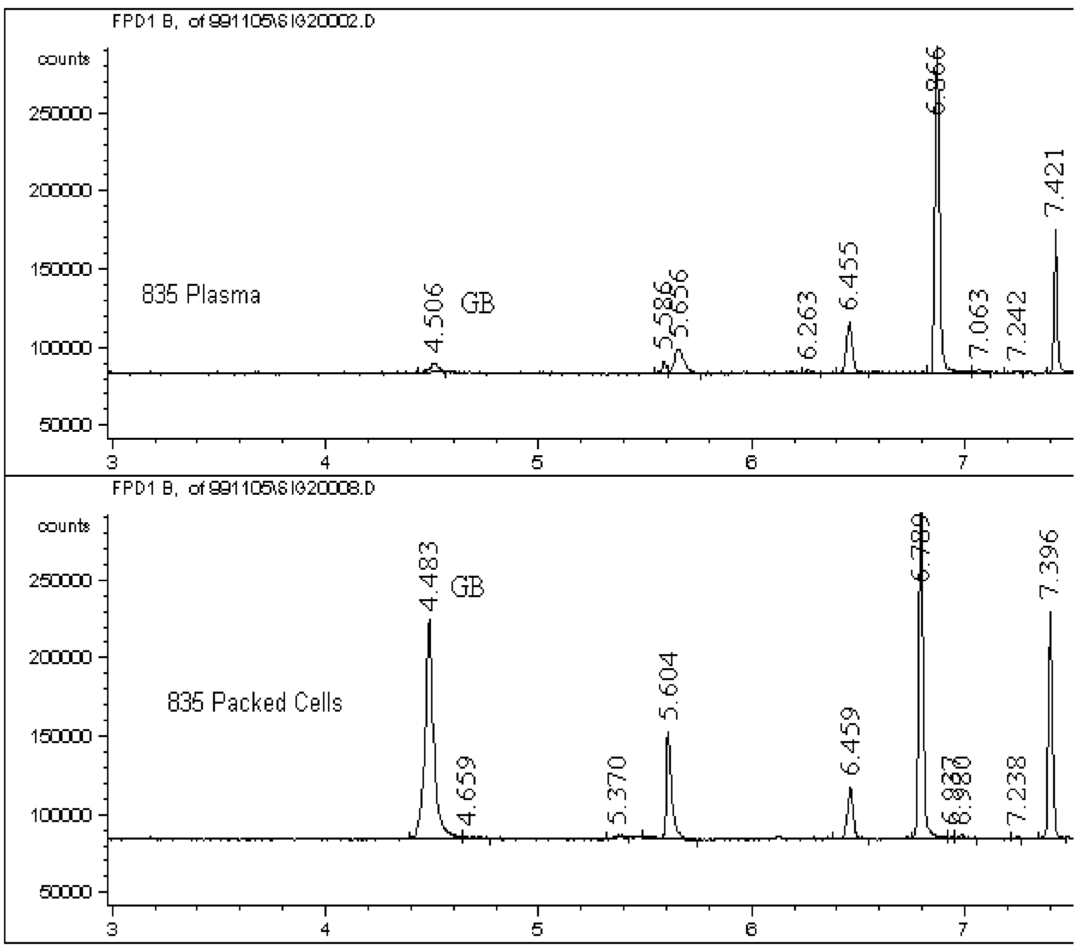

Figure 2. Chromatograms Comparing Response for GB in Plasma and Packed Cell Samples (Ct=1076mg min/m^3).

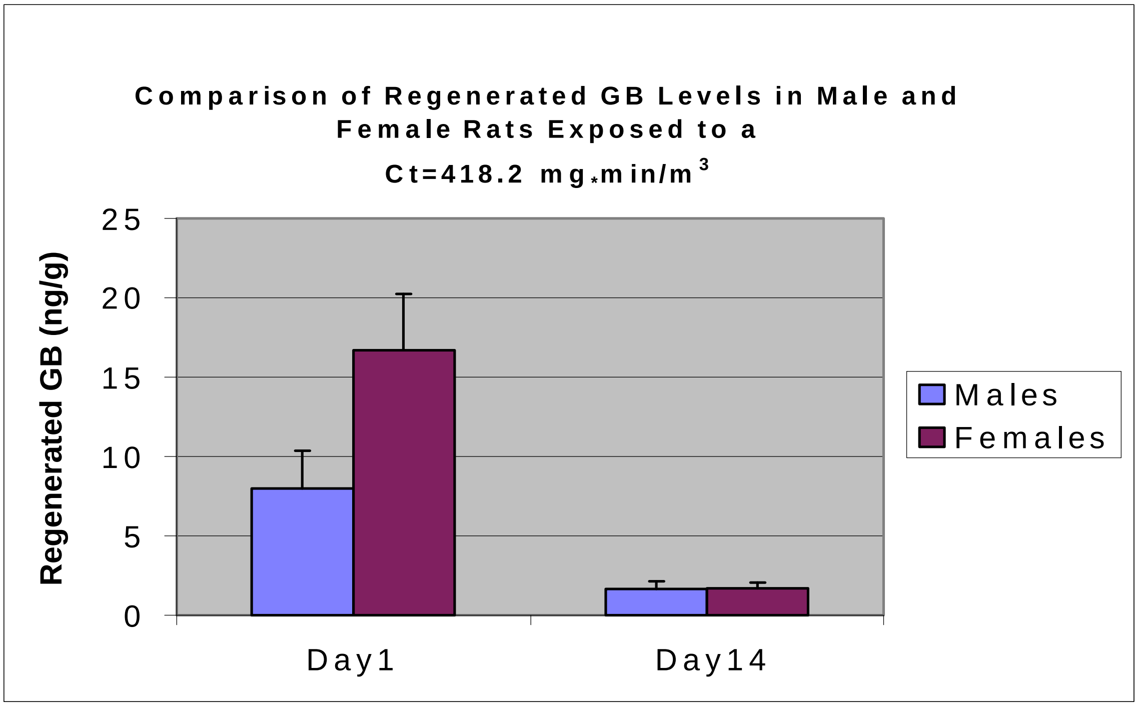

Figure 3. Comparison of GB Levels (6 hr test, Ct=418.2 mg min/m3): Day 1 *vs* Day 14.

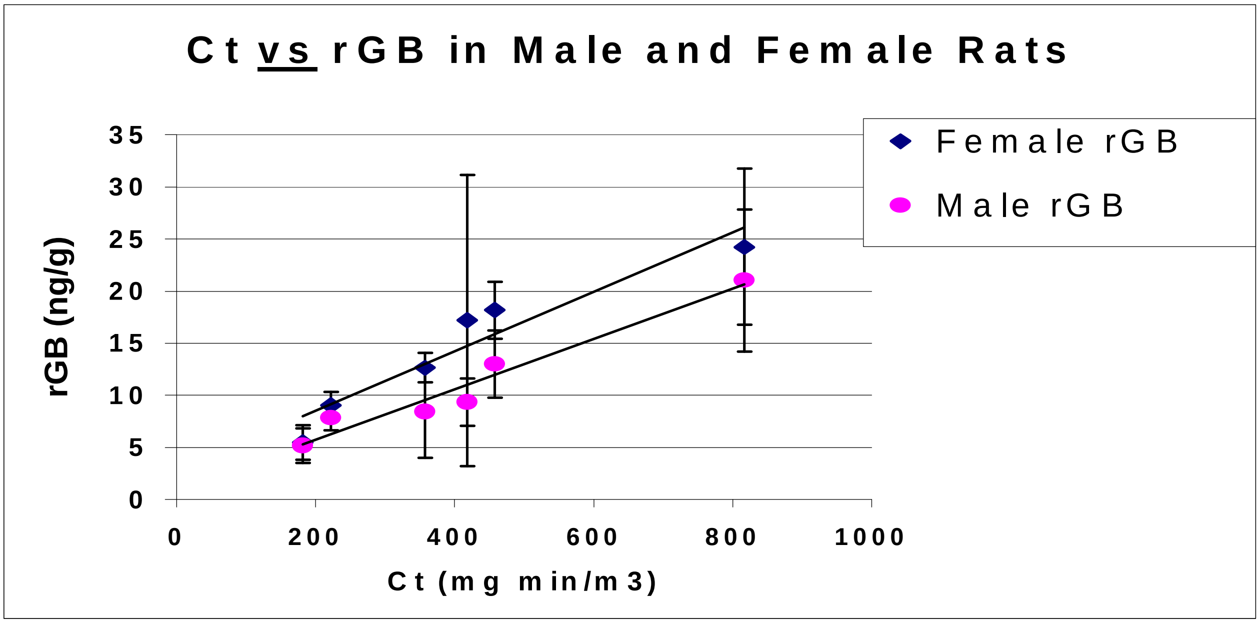

Figure 4. Ct versus rGB in male and female rats after inhalation exposure.

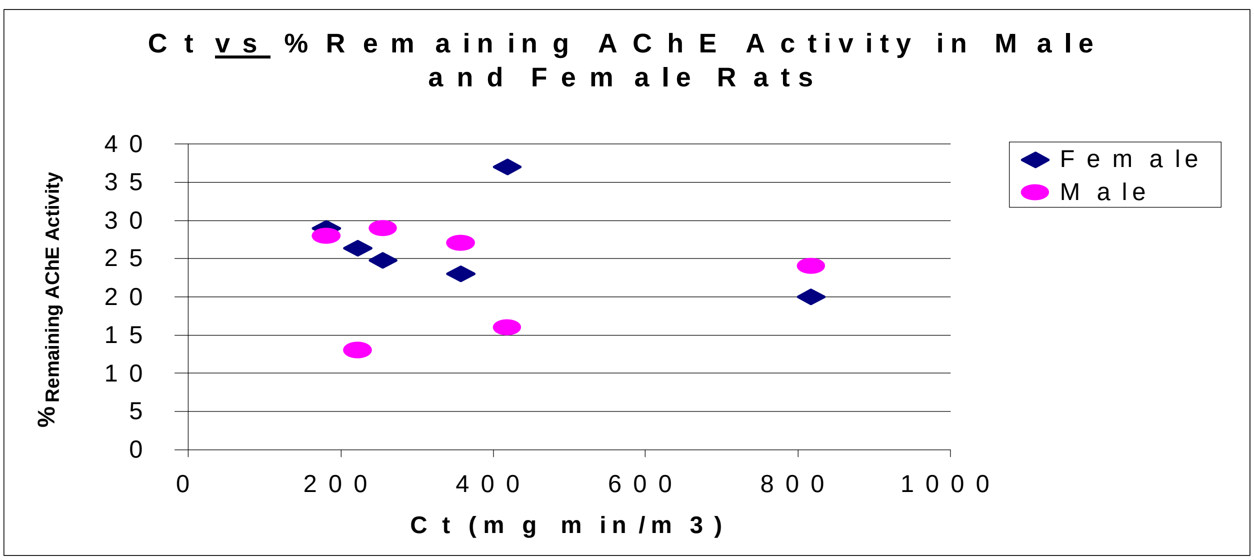

Figure 5. Ct versus AchE activity in male and female rats after inhalation exposure.